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14. ABSTRACT
EphB4 is a member of the Eph family of receptor tyrosine kinases that is widely expressed in many cancer cell types. High expression of EphB4 has been positively correlated with prostate cancer malignancy. On the other hand, EphB4 has also been shown to be downregulated in other types of cancer. It is unclear how this receptor may promote or suppress oncogenesis under different circumstances. One possibility would be the Eph receptors have oncogenic activity when ephrin stimulation is low or absent, whereas activation of downstream signaling by high levels of these ligands suppresses the malignant properties of prostate cancer cells. I have shown that ephrin-A1 stimulation of EphA2, another Eph receptor widely expressed in cancer, triggers inactivation of the anti-oncogenic Akt-mTOR pathway through crosstalk with a serine/threonine phosphatase. The resulting tumor-suppressing effects could be exploited for prostate cancer therapy if the responsible signaling pathways could be maintained for prolonged periods. However, ephrin stimulation also causes rapid degradation of the Eph receptors, which in turn terminates their anti-oncogenic activities. Therefore, understanding the mechanisms through which E3 ubiquitin ligases regulate Eph receptor stability will help potentiate the anti-oncogenic effects of Eph receptors. I have found that two E3 ubiquitin ligases, Cbl and RNF5, seems not to be major ubiquitin ligases responsible for regulating Eph receptor stability in PC3 prostate cancer cells. On the other hand, the E3 ubiquitin ligase complex Cul4B-DDB1-DCAF5 identified by mass spectrometry as a possible ephrin-A1/EphA2 binding partner, may regulate EphA2 stability. Preventing Eph receptor degradation, for example by inhibiting ubiquitin ligases, should enhance Eph receptor-dependent anti-oncogenic signaling and thus represents a promising strategy for the design of novel therapeutic strategies.

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Introduction

Loss of function of tumor suppressor genes and gain of function of tumor-promoting genes are critical steps in the development and progression of cancer. It is therefore important to identify these genes and understand how they function in order to develop new treatments. The EphB4 receptor tyrosine kinase has been reported to be upregulated in various types of cancer. In human prostate cancers, EphB4 has been found in the majority of tumor specimens examined (1). On the other hand, knocking down EphB4 with antisense oligonucleotides or siRNA appears to have anti-tumor activity in prostate, breast and bladder cancer cells grown in culture and in vivo in tumor xenografts (1-3), suggesting that this receptor plays an important role in tumorigenesis. However, there are also some reports showing that EphB4 expression is negatively correlated to tumor growth in breast cancer cells (4) and downregulated in the more advanced stages of colon cancer (5). Therefore, more investigations are needed to elucidate the role of Eph receptors in cancer and to determine how these receptors can have tumor promoter or tumor suppressor activities depending on the conditions.

Our laboratory has previously shown that activation of EphB4 by its ligand, ephrin-B2, can widely inhibit the malignant properties of breast cancer cells through an EphB4 signaling pathway that involves activation of the Abl tyrosine kinase and inhibition of the proto-oncogene Crk (6). Moreover, ligand stimulation of EphA2, another Eph receptor highly expressed in prostate cancer cells (7, 8) and recently identified as a prostate cancer biomarker (9), has been reported to inhibit the Ras-MAP kinase oncogenic pathway (10). My work supported by this award has also led to the identification of a novel tumor suppressor pathway triggered by Eph receptors upon ephrin stimulation in prostate cancer cells. This tumor suppressor pathway involves inhibition of the Akt-mTORC1 signaling axis, an important oncogenic signaling pathway often hyperactivated in prostate cancer cells due to various mutations (11). The fact that the ephrin ligands are generally low or absent in prostate cancer cells leads to my hypothesis that the Eph receptors have oncogenic activity when ephrin stimulation is low or absent, whereas activation of downstream signaling by high levels of these ligands suppresses the malignant properties of prostate cancer cells.

Although the ephrin-induced activation of Eph receptors mentioned above could represent a powerful anti-oncogenic tool, the effects are short-lived due to the rapid ephrin-induced degradation of Eph receptors, which seems to partially depend on ubiquitination and the proteasome. Therefore, this report is mainly focused on investigation of E3 ubiquitin ligases involved in Eph receptors degradation in order to find approaches to potentiate the anti-oncogenic effects of Eph receptors by inhibiting their degradation.

Aim 1. Determine how EphB4 expression and signaling affect prostate cancer cell behavior

Task 1. Perform EphB4 Y837F mutant transfection and siRNA experiments to investigate whether Cbl is the main E3 ubiquitin ligase responsible for ephrin-mediated degradation of Eph receptors

Treatment of PC3 cells with ephrin-B2 Fc results in substantial EphB4 downregulation. Similarly, treating PC3 cells with ephrin-A1 Fc also causes drastic reduction of EphA2 levels, (Fig.1 and as described in the 2008 DOD application). Recent studies have implicated the ubiquitin ligase Cbl in ephrin-mediated downregulation of several Eph receptors, including EphA2 and EphB1 (12-14). I have engineered an EphB4 Y837F mutant, which could potentially block the interaction between EphB4 and Cbl because this tyrosine is in a similar consensus motif (DpYRLP) as tyrosine 1003 of the Met receptor tyrosine kinase and when phosphorylated

mediates Cbl binding (15). Although the EphB4 Y837F mutant successfully abolishes the binding between EphB4 and Cbl in pull-down experiments (as described in the 2010 progress report), it does not seem to have a significant impact on ephrin-B2 mediated degradation of EphB4 (data not shown). This lack of detectable effect of the EphB4 Y837F mutation on EphB4 degradation could be due to low transfection efficiency (about 30-40%) and, therefore, masking of the mutant stability by high levels of endogenous wild-type EphB4 in PC3 cells. To further investigate the role of Cbl in ephrin-B2-mediated degradation of EphB4, I also performed siRNA-mediated knockdown of Cbl. However, I have not been able to knock down Cbl levels sufficiently, despite using high concentrations (up to 200 nM) of different Cbl siRNAs. Therefore, stable transfection of the EphB4 Y837F mutant or introduction of Cbl shRNA by lentivirus infection in PC3 cells will be performed to obtain more conclusive results.

Task 2. Identify other E3 ubiquitin ligases involved in ephrin-induced degradation of Eph receptors

Our laboratory recently used 1D LC/MS/MS mass spectrometry to identify the Eph receptor expressed in PC3 prostate cancer cells. We used the ligand ephrin-A1 or ephrin-B2 fused to the Fc portion of human IgG and immobilized on protein A beads in pulldown experiments. Ephrin-associated proteins were separated on SDS-PAGE gels and silver-stained. The portion of the gel including the 80-180 KDa region was incubated with trypsin, followed by mass spectrometry. Based on spectral counts, the EphA2 and EphB4 receptors were the most abundant proteins identified in the ephrin-A1 or ephrin-B2 pull-down experiments, respectively. No EphA2 or EphB4 peptides were identified in the control Fc pulldowns. These data show that EphA2 and EphB4 are the major Eph receptors present in PC3 cells. Interestingly, a number of other proteins were presented in the ephrin-A1 Fc but not in the ephrin-B or the control Fc pulldowns. These proteins are candidate EphA2-interacting protein. Cullin 4B (Cul4B), DDB1 and DCAF5 (Table 1) caught our attention because they are known to form a complex (16, 17). Cul4B is an E3 ubiquitin ligase, DDB1 is an adaptor that links substrate receptors with Cul4B, and DCAF5 (also known as WDR22) is a substrate receptor that can bind to the Cul4B-DDB1 complex. Notably, no other Cullins were detected in these experiments.

To investigate whether the Cul4B-DDB1-DCAF5 complex regulates EphA2, I co-transfected EphA2, Cul4B and HA-ubiquitin in 293T cells. EphA2 was then immunoprecipitated and its ubiquitination was detected by immunoblotting with anti-HA antibodies. These experiments revealed that EphA2 is much more ubiquitinated in the Cul4B-transfected cells compared to the vector-transfected cells (Figure 2), suggesting that EphA2 might be a substrate for Cul4B. Whether similar effects are observed in PC3 prostate cancer cells will be determined next. To further investigate whether the Cullin family affects ephrin-A1-mediated degradation of EphA2, PC3 cells were pretreated with an inhibitor of Cullin activity, MLN4924, followed by ephrin-A1 Fc stimulation. The EphA2 levels did not appear to be different between the control DMSO and MLN4924-treated samples, suggesting that the Cullin family is not involved in ephrin-A1-induced degradation of EphA2 (Figure 3). However, Cullins may be involved in EphA2 degradation in the absence of ephrin-A1 (see above). Introduction of Cul4B siRNA in PC3 cells is a complementary approach that will also help establish the importance of Cul4B in the regulation of Eph receptors. On the other hand, it is also possible that EphA2 may regulate the activity of Cul4B-DDB1-DCAF5 complex and thus degradation of other proteins by the complex. Therefore, it will be important in the future to assay EphA2-mediated phosphorylation

of individual proteins in the Cul4B-DDB1-DCAF5 complex as well as whether EphA2 activation affects ubiquitination of Cul4B substrates.

I have also investigated a possible role of RNF5, another E3 ubiquitin ligase, in the regulation of EphA2 levels in PC3 cells. The Ronai laboratory at our institute obtained evidence that EphA2 may be an RNF5 target. However, co-transfection of EphA2, RNF-5 and HA-ubiquitin in 293T cells followed by EphA2 immunoprecipitation did not show increased EphA2 ubiquitination compared to the control transfection in which RNF5 was omitted. Furthermore, EphA2 stability in the presence or in the absence of the ephrin-A1 ligand was not different between the wild-type and RNF5 knockout PC3 cells. Similar results were also obtained in RNF5 knockout mouse embryonic fibroblasts and HeLa cells, indicating that RNF5 is not a major E3 ubiquitin ligase responsible for regulating EphA2 stability. However, whether EphA2 may regulate RNF5 activities remains to be determined.

Aim 2. Characterize EphB4 signaling mechanisms in prostate cancer cells

Task 1. Examine signaling pathways modulated by exogenous ephrin stimulation of Eph receptors in prostate cancer cells

To explore the signaling pathways activated downstream of Eph receptors following ephrin stimulation of PC3 prostate cancer cells, I utilized the antibody-based Human Phospho-Kinase Array Kit from R&D Systems. Because the robust dephosphorylation of Akt downstream of EphA2 can serve as a positive control (Yang et al. 2010), I investigated ephrin-A1-mediated signaling downstream of EphA2 first. Lysates from PC3 cells stimulated with ephrin-A1 Fc or control Fc were analyzed using the kit, which can detect 46 kinase phosphorylation sites. Several kinases appeared to be significantly dephosphorylated following ephrin-A1 stimulation, including my positive control Akt (which was dephosphorylated on both threonine 308 and serine 473), p70S6 kinase, members of the STAT family, FAK, beta-catenin and AMPK2 (Figure 4). I therefore performed immunoprecipitations and immunoblotting to verify the changes in phosphorylation of some of the candidate proteins. However, only dephosphorylation of p70S6 kinase, in addition to the Akt positive controls, was confirmed. Dephosphorylation of STAT family proteins, FAK and paxillin was not detectable in the immunoprecipitated proteins (data not shown).

Key Research Accomplishments

1. I determined that the EphB4 Y837F mutant is defective in Cbl E3 ubiquitin ligase binding.
2. I obtained preliminary data suggesting that Cbl may not play a major role in ephrin-B2-mediated degradation of EphB4 in PC3 prostate cancer.
3. I discovered a possible interplay between the Cullin 4B E3 ubiquitin ligase, which may be involved in regulating EphA2 ubiquitination and stability.
4. I obtained preliminary results suggesting that the RNF5 E3 ubiquitin ligase does not play a major role in EphA2 degradation upon ephrin-A1 stimulation of PC3 prostate cancer cells.
5. I found that the STAT family, FAK and paxillin are not likely to be robust downstream effectors of EphA2 upon ephrin-A1 stimulation of PC3 prostate cancer cells.

Reportable Outcomes

Papers

Yang NY, Fernandez C, Richter M, Xiao Z, Valencia F, Tice DA, Pasquale EB (2011). Crosstalk of the EphA2 receptor with a serine/threonine phosphatase suppresses the Akt-mTORC1 pathway in cancer cells. Cell Signaling 23:201-212.

Presentations

Poster, ”EphA2 and EphB4 in prostate cancer’’ presented at the Innovative Minds in Prostate Cancer Today (IMPaCT) Conference 2011, Orlando, FL.

Classes

1. Biomarkers, UCSD extension
2. Stem Cell Biology, UCSD extension

Conclusions

The work I have performed last year has been focused on investigating E3 ubiquitin ligases involved in regulation of Eph receptor stability in prostate cancer cells. While ephrin stimulation of Eph receptors triggers several powerful tumor-suppressing signaling pathways, ephrins also cause rapid degradation of Eph receptors, which in turn terminates their anti-oncogenic activities. Therefore, understanding the mechanisms through which E3 ubiquitin ligases regulate Eph receptor stability will help to potentiate the anti-oncogenic effects of Eph receptors. I have found that Cbl, a E3 ubiquitine ligase well-known for regulating many receptor tyrosine kinases, seems not to be the major ubiquitin ligase responsible for EphB4 degradation in PC3 prostate cancer cells exposed to the ligand, ephrin-B2. In addition, another E3 ubiquitine ligase, RNF5, seems not to be the major ubiquitine ligase responsible for regulating EphA2 stability in the presence of ephrin-A1. On the other hand, the E3 ligase complex Cul4B-DDB1-DCAF5 identified by mass spectrometry may regulate EphA2 stability. Investigation of other signaling pathways downstream of Eph receptors upon ephrin stimulation has also been ongoing and I have found that the STAT family, FAK and paxillin are not major downstream effectors of EphA2 after ephrin-A1 treatment, in contrast to Akt and p70S6 kinase. Taken together, my results show that promoting Eph receptor signaling could represent a powerful approach in order to suppress prostate cancer progression. Understanding how to maintain the Eph receptor-dependent anti-oncogenic effects for prolonged periods by preventing receptor degradation is a promising strategy for the design of novel anti-prostate cancer therapies.

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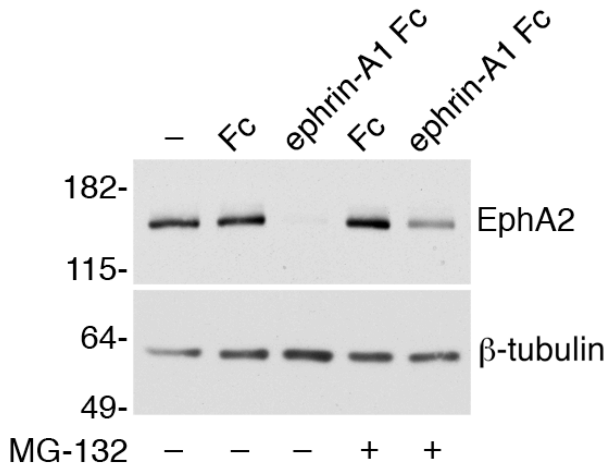

Fig. 1. Ephrin-A1 causes EphA2 degradation and the effect can be inhibited by the proteasome inhibitor MG-132. PC3 cells were treated with the ephrin-A1 Fc ligand for 2 hours with and without MG-132. EphA2 levels were examined by immunoblotting.

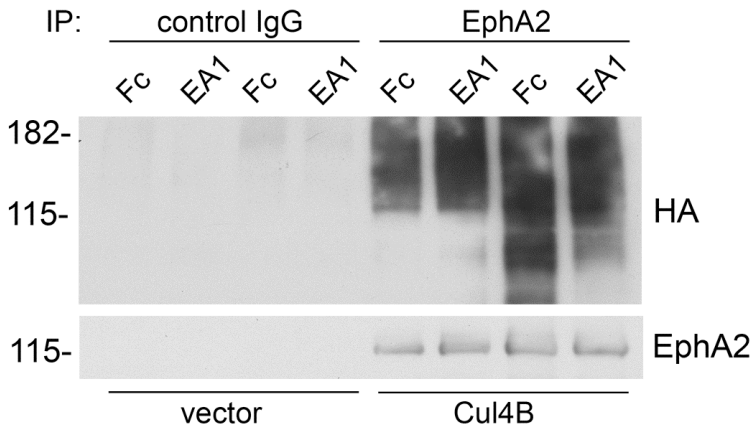

Fig. 2. EphA2 may be a substrate for the Cul4B E3 ubiquitine ligase. HEK293T cells were co-transfected with EphA2, HA-ubiquitin, and Cul4B (or control vector). 48 hours after transfection, the cells were stimulated with 1 μg/ml ephrin-A1 Fc or control Fc. Cell lysates were then immunoprecipitated with anti-EphA2 antibodies or control IgG and ubiquitination was detected with anti-HA antibodies.

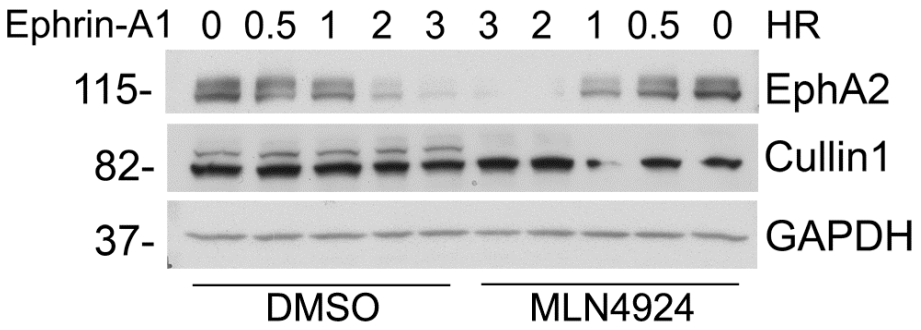

Fig. 3. Cul4B does not appear to be the major E3 ubiquitin ligase responsible for EphA2 degradation following ephrin-A1 stimulation. PC3 cells were pretreated with DMSO or 1 μM MLN4924 Cullin inhibitor for 24 hours, followed by stimulation of 1 μg/ml ephrin-A1 Fc for the indicated times. Cell lysates were immunoblotted with the indicated antibodies. Cullin 1 signals serve as a control for MLN4924 inhibitory activity: the upper Cullin 1 band represents the neddylated form of the protein and can be inhibited by the treatment of MLN4924.

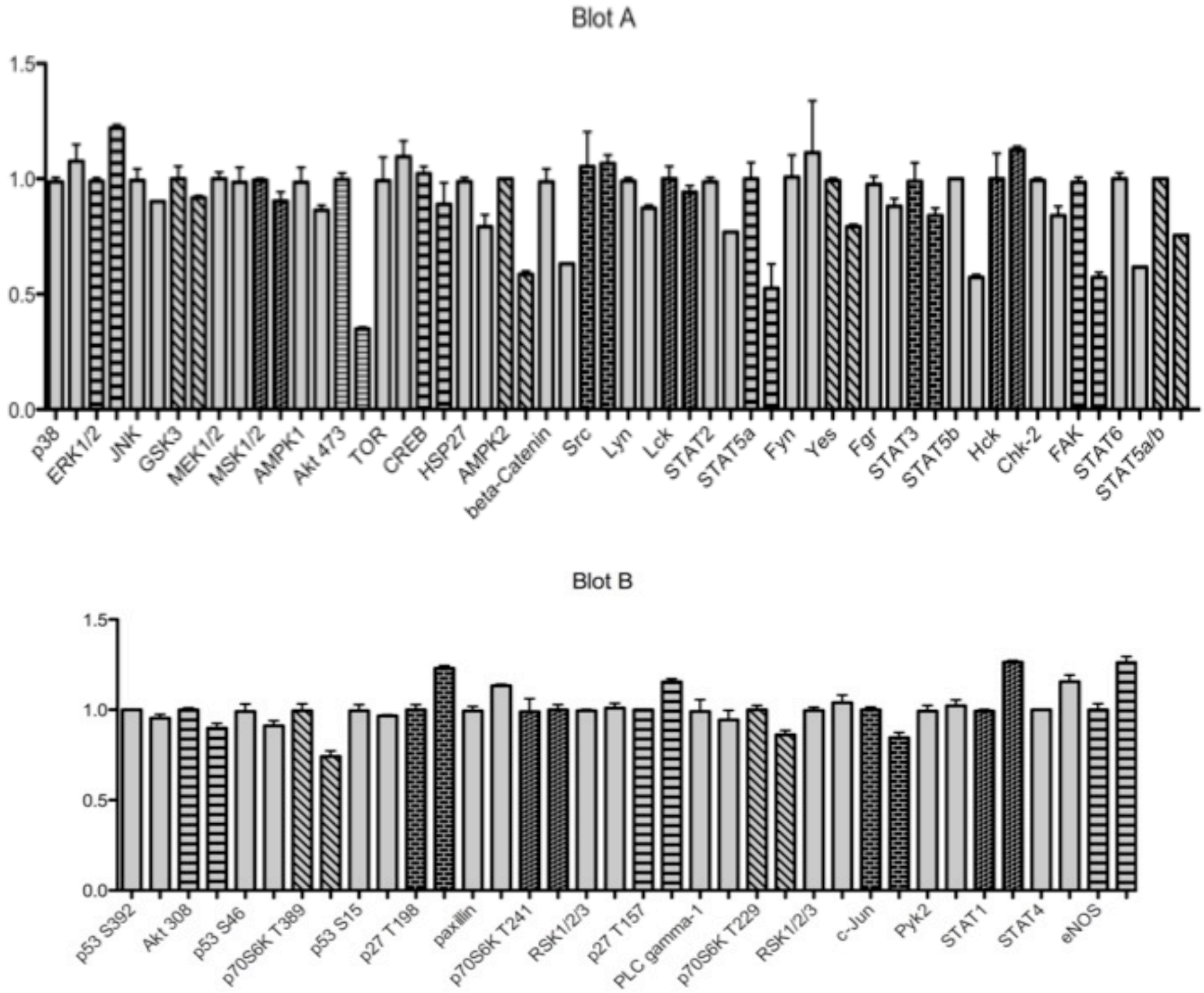

Fig. 4. Possible downstream effector kinases of EphA2 following ephrin-A1 stimulation. The antibody-based Human Phospho-Kinase Array Kit from R&D Systems was used to measure phosphorylation of cytoplasmic kinases in PC3 cells exposed to eprhin-A1 Fc for 20 min. The histograms show averages ± SEM for phosphorylation of individual kinases in cells treated with control Fc (first bar) or ephrin-A1 Fc (second bar).

Table 1. Mass spectrometry results for Eph receptors and Cul4B complex

Pull-down		EphA2 or EphB4	Cul4B	DDB1	DCAF5
Fc #1	Spectral count	0	0	21	0
	Peptide coverage	0	0	11.8	0
Ephrin-A1 Fc #1	Spectral count	268	18	59	34
	Peptide coverage	50.4	14.4	23.9	16.6
Fc #2	Spectral count	0	0	8	0
	Peptide coverage	0	0	5	0
Ephrin-A1 Fc #2	Spectral count	156	3	36	14
	Peptide coverage	38.9	4.7	13.5	11.1
Ephrin-B2 Fc	Spectral count	16	0	8	1
	Peptide coverage	9.4	0	7	4.3

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Crosstalk of the EphA2 receptor with a serine/threonine phosphatase suppresses the Akt-mTORC1 pathway in cancer cells

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ABSTRACT

Receptor tyrosine kinases of the Eph family play multiple roles in the physiological regulation of tissue homeostasis and in the pathogenesis of various diseases, including cancer. The EphA2 receptor is highly expressed in most cancer cell types, where it has disparate activities that are not well understood. It has been reported that interplay of EphA2 with oncogenic signaling pathways promotes cancer cell malignancy independently of ephrin ligand binding and receptor kinase activity. In contrast, stimulation of EphA2 signaling with ephrin-A ligands can suppress malignancy by inhibiting the Ras-MAP kinase pathway, integrin-mediated adhesion, and epithelial to mesenchymal transition. Here we show that ephrin-A1 ligand-dependent activation of EphA2 decreases the growth of PC3 prostate cancer cells and profoundly inhibits the Akt-mTORC1 pathway, which is hyperactivated due to loss of the PTEN tumor suppressor. Our results do not implicate changes in the activity of Akt upstream regulators (such as Ras family GTPases, PI3 kinase, integrins, or the Ship2 lipid phosphatase) in the observed loss of Akt T308 and S473 phosphorylation downstream of EphA2. Indeed, EphA2 can inhibit Akt phosphorylation induced by oncogenic mutations of not only PTEN but also PI3 kinase. Furthermore, it can decrease the hyperphosphorylation induced by constitutive membrane-targeting of Akt. Our data suggest a novel signaling mechanism whereby EphA2 inactivates the Akt-mTORC1 oncogenic pathway through Akt dephosphorylation mediated by a serine/threonine phosphatase. Ephrin-A1-induced Akt dephosphorylation was observed not only in PC3 prostate cancer cells but also in other cancer cell types. Thus, activation of EphA2 signaling represents a possible new avenue for anti-cancer therapies that exploit the remarkable ability of this receptor to counteract multiple oncogenic signaling pathways.

1. Introduction

The serine/threonine kinase mTOR (mammalian Target of Rapamycin), which is of major importance for cell growth, has recently received much attention as a possible novel target for anti-cancer drugs [1–4]. mTOR functions downstream of the serine/threonine kinase Akt as part of the mTOR complex 1 (mTORC1) protein complex and upstream of Akt as part of the mTORC2 complex [5–8]. Typically, growth factor receptors activate Akt through PI3 kinase, which phosphorylates the phospholipid PI(4,5)P2 to produce PI(3,4,5)P3. Binding to PI(3,4,5)P3 causes relocalization of Akt to the plasma membrane. Here, Akt is activated through phosphorylation at T308 by the PDK1 kinase, which is also anchored to the plasma membrane by PI(3,4,5)P3, and through phosphorylation at S473 by mTORC2. Activated Akt in turn phosphorylates and inactivates Tuberous sclerosis complex 2 (TSC2), which is a GTPase-activating protein for the Ras family protein Rheb. This leads to activation of Rheb and its downstream target mTORC1. Two major downstream targets of mTORC1 that regulate mRNA translation are the 4E-BP translational repressor and S6 kinase, which phosphorylates the S6 ribosomal protein to promote protein synthesis. The Akt-mTORC1 pathway is often activated in cancer cells due to loss of the tumor suppressor PTEN, a lipid phosphatase that dephosphorylates PI(3,4,5)P3 to PI(4,5)P2 [9,10]. PTEN loss is prevalent in prostate cancer, and reducing PTEN levels in mouse prostate epithelial cells is sufficient to induce cancer development through hyperactivation of Akt and mTORC1 [11–13]. Activating mutations in PI3 kinase or Akt, and deregulation of growth factor receptors, can also result in activation of

Abbreviations: MAP kinase, mitogen-activated protein kinase; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PI(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; PI3K, phosphatidylinositol 3 kinase; PDK1, phosphoinositide-dependent kinase 1; PHLPP, PH domain and leucine-rich repeat protein phosphatase; PP, protein phosphatase; PTEN, phosphatase and tensin homolog; mTORC1, mTOR complex 1; mTORC2, mTOR complex 2; TSC2, Tuberous sclerosis complex 2.

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the Akt-mTORC1 pathway in cancer cells [10,14]. This pathway can promote cancer cell growth as well as migration and invasiveness, and often cooperates with the Ras-MAP kinase pathway to induce malignant transformation [6,15,16].

Receptor tyrosine kinases of the Eph family can suppress cancer cell growth, migration and invasiveness through multiple signaling pathways activated by ephrin ligands and whose underlying mechanisms are not completely understood [17]. Eph receptors can, for example, inhibit the Ras-MAP kinase pathway [18,19], the Crk proto-oncogene [20–22], integrin-mediated adhesion [21,23–25], and epithelial–mesenchymal transition [26,27]. Furthermore, a recent report has shown that the EphA2 receptor can also inhibit Akt phosphorylation in cancer cells, but the molecular mechanisms involved and the downstream pathways affected were not elucidated [28]. Here we show that ligand-dependent activation of EphA2 decreases the growth of PC3 prostate cancer cells, which lack PTEN [29]. In these and other cancer cell types, ephrin-A1 stimulation inactivates the Akt-mTORC1 pathway. Our results suggest that Akt dephosphorylation downstream of ligand-activated EphA2 depends on a novel mechanism involving crosstalk with a serine/threonine phosphatase.

2. Materials and methods

2.1. Cell lines and transfections

PC3 and WM793 cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS); Lu1205, UACC903, and HT-29 cells in DMEM medium with 10% FBS; SKOV-3 cells in McCoy's 5a modified medium with 10% FBS; MDA-MB-231 cells in DMEM/F12 medium with 10% FBS; MCF-10A cells in DMEM/F12 medium with 10 ng/ml VEGF, 5 μg/ml insulin and 5% FBS.

siGENOME SMARTpool siRNAs (Dharmacon) were used for knockdown of EphA2, Ship2, PHLPP1 and PHLPP2. Dharmacon siCONTROL non-targeting siRNA, which engages the RISC complex but does not target any mouse or human genes, was used as a control. The siRNA transfection protocol was optimized for PC3 cells. The cells were transfected with 40 nM EphA2 siRNA, 80 nM Ship2 siRNA, or 62 nM PHLPP1 and 62 nM PHLPP2 siRNAs using Lipofectamine 2000 or Lipofectamine RNAiMax (Invitrogen Life Technologies) according to the manufacturer's instructions. The cells were then stimulated with ephrins or antibodies 2 days after transfection.

For plasmid transfections in PC3 cells, cells in 60 mm plates were transfected with 2 μg total plasmid DNA and 8 μl Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. The plasmids used include: pcDNA3 vector, pCMV vector, HA-tagged wild-type and myristoylated Akt1 in pcDNA3, wild-type and constitutively active CAAX-PI3 kinase in pcDNA3, wild-type and constitutively active H-Ras G12V in pcDNA3, wild-type and constitutively active R-Ras G38VY66F in pcDNA3. Cells were used 2 days after transfection.

2.2. Cell growth measurements

PC3 cells grown in a medium containing 10% FBS were stimulated with unclustered ephrin-A1 Fc or Fc as a control, or left unstimulated. In some cases, the cells were also treated with various inhibitors, including LY294002 (Promega, 3 mM stock dissolved in DMSO); PD98059 (LC Laboratories, 20 mM stock dissolved in DMSO); and rapamycin (LC Laboratories, 50 mM stock dissolved in ethanol). For 2D growth on tissue culture plates, cells were counted in a hemocytometer or viable cells were quantified using the MTT assay (Calbiochem). Briefly, 5000 cells/well were seeded in 96-well plates, allowed to attach, and then treated with the various inhibitors. For focus formation assays, cells plated at low density were grown for 11 days and stained with crystal violet. The plates were scanned to visualize the foci and then the cells were solubilized and the absorbance at 570 nm was measured. To measure 3D growth in Matrigel (BD Bioscience), cells were plated at low density and spheroids were photographed at different time points. Spheroid volume was estimated as $(d\ max \times d\ min^2 \times \pi)/6$ (d = diameter). To measure 3D growth in soft agar, cells were plated at low density in 6-well plates on 1.5 ml 0.5% low melting agarose (Gibco) and covered with 1 ml 0.3% low melting agarose. After 3 weeks, the wells were photographed under a 10× objective and colonies were counted from 10 photographs per condition (5 photographs/well, 2 wells/experiment).

2.3. Immunoblotting and immunoprecipitation

For ephrin stimulation experiments, cells grown in 10% FBS were stimulated with ephrin Fc fusion proteins (R&D Systems), which in some cases were preclustered with 1/10 concentration of goat anti-human IgG antibody (Jackson ImmunoResearch). For stimulation with immobilized ephrin-A1 Fc, PC3 cells were allowed to attach for 15 min on Petri dishes coated with 3 μg/ml ephrin-A1 Fc or Fc as a control. In the experiment shown in Fig. 7B, some of the cells were grown overnight in the absence of FBS before stimulation. In some experiments, cells were pre-treated with calyculin (Calbiochem/EMB Bioscience; 20 μM stock in DMSO), tautomycin (Calbiochem/EMB Bioscience, 1 mM stock in ethanol), okadaic acid (MP Biomedicals, 150 μM stock in DMSO), LY294002, PD98059, rapamycin (see previous section), dasatinib (LC Laboratories; 50 μM stock in DMSO), PP2 (Calbiochem/EMB Bioscience, 10 mM stock in DMSO, or Gleevec (LC Laboratories, 10 mM stock in DMSO).

For immunoblotting, ephrin-stimulated cells and cells transfected with siRNAs or plasmids were lysed in modified RIPA buffer (50 mM TrisHCl, pH 7.6, 150 mM NaCl, 1% TritonX-100, 0.5% sodium deoxycholate, 0.1% SDS, 2 mM EDTA with protease and phosphatase inhibitors) and analyzed by SDS-PAGE followed by immunoblotting with various primary antibodies. Antibodies to phospho-TSC2 T1462, phospho-Akt T308, phospho-Akt S473, Akt, phospho-threonine, phospho-Erk T202/Y204, Erk1/2, phospho-S6 kinase T389, S6 kinase, phospho-Src, phospho-Crk and Ship2 were from Cell Signaling Technology; antibodies to TSC2 and R-Ras were from Santa Cruz Biotechnology; the 9EG7 anti-β1 integrin-activating antibody, the anti-Crk antibody, the anti-phosphotyrosine antibody conjugated to horseradish peroxidase (HRP) and the anti-Ras antibody were from BD Biosciences; antibodies to PHLPP1 and PHLPP2 were from Bethyl Laboratories; antibodies to EphA2 were from Zymed/Invitrogen (polyclonal, used for immunoblotting) and Upstate Biotechnology/Millipore (monoclonal D7, used for immunoprecipitation); the EphA4 monoclonal antibody was from Zymed/Invitrogen; the anti-Src antibody and goat anti-rabbit and sheep anti-mouse secondary antibodies conjugated to HRP were from Millipore.

For immunoprecipitations, PC3 cells were lysed in modified RIPA buffer and EphA2 was immunoprecipitated with 2.5 μg anti-EphA2 monoclonal antibody (Upstate Biotechnology/Millipore) bound to GammaBind Plus sepharose beads (GE Healthcare). The immunoprecipitates were separated by SDS-PAGE and probed by immunoblotting with an anti-phosphotyrosine or anti-phospho-Akt substrate antibody and reprobed with an anti-EphA2 polyclonal antibody. Other cell types used for immunoprecipitations were lysed in HEPES buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 1% NP-40, 0.25% Na deoxycholate with phosphatase and protease inhibitors (Sigma)) and Eph receptors were immunoprecipitated using 2 μg anti-EphA2 or anti-EphA4 antibodies bound to 15 μl anti-mouse IgG beads (Sigma).

2.4. MesoScale quantification

This was carried out with a pAktS473 assay kit (Meso Scale Discovery) according to the manufacturer's instructions.

2.5. Measurement of integrin signaling and cell retraction

To examine the effects of integrin-mediated adhesion on Akt phosphorylation, PC3 and WM793 cells were trypsinized, washed in

DMEM containing 1% BSA and kept in suspension at 37 °C in the same medium for 30 min. Cells (1.5×10^6 cells per 60 mm plate) were then allowed to attach for 15 min at 37 °C to plates coated with poly-L-lysine (10 μg/ml for PC3 cells and 5 μg/ml WM793 cells), 10 μg/ml fibronectin (for PC3 cells), or 2.5 μg/ml vitronectin (for WM793 cells) in the presence of 7.5 μg/ml preclustered ephrin-A1 Fc or control Fc. To determine Akt phosphorylation, both adherent and non-adherent cells were pooled, lysed in HEPES buffer and probed by immunoblotting.

To measure the effects of maintaining integrin activity with Mn^{2+} on ephrin-A1-dependent Akt inactivation, PC3 and WM793 cells were seeded on non-coated tissue culture plates the day before the experiment. Sub-confluent cultures were pre-treated with 1 mM Mn^{2+} for 30 min at 37 °C before they were stimulated for 30 min at 37 °C with 4 μg/ml preclustered ephrin-A1 Fc or control Fc. To measure the effects of maintaining integrin activity with an integrin-activating antibody, PC3 and WM793 cells were plated on 10 μg/ml fibronectin overnight at 37 °C. Cells were washed in pre-warmed PBS before incubation with 20 μg/ml 9EG7 anti-β1 integrin-activating antibody in RPMI for 30 min at 37 °C. Cells were then stimulated for 30 min at 37 °C with 4 μg/ml preclustered ephrin-A1 Fc or control Fc followed by a washing step with pre-warmed PBS and cell lysis in HEPES buffer.

To confirm inhibition of ephrin-A1-induced integrin inactivation, PC3 and WM793 cells (3×10^4 cells per well of a 24-well plate) were plated on coverslips coated with 5 μg/ml poly-L-lysine or 10 μg/ml fibronectin and pre-treated either with Mn^{2+} or anti-β1 integrin antibodies before ephrin-A1 Fc or control Fc stimulation. Cells were then fixed in 4% paraformaldehyde and stained with DAPI and FITC-

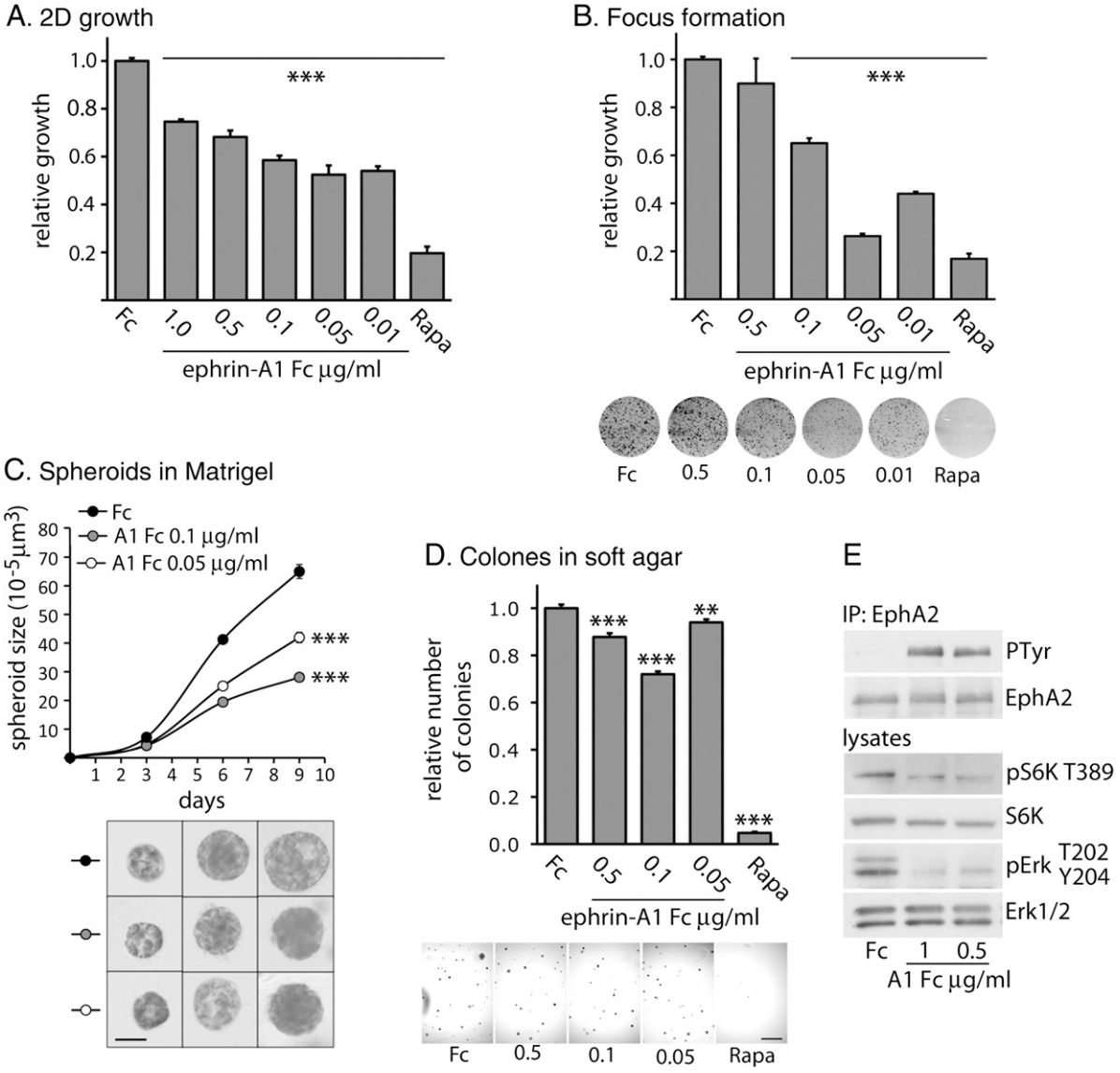

Fig. 1. EphA2 activation by ephrin-A1 inhibits PC3 cell growth. (A) Two-dimensional growth on tissue culture plates. Cells were grown in a medium containing the indicated concentrations of ephrin-A1 Fc, 0.1 μg/ml control Fc, or 100 nM rapamycin, and counted after 3 days. The histogram shows average relative cell growth ± SE from 2–6 experiments with triplicate measurements. (B) Focus formation assays. Cells were grown for 11 days in a medium containing the indicated concentrations of ephrin-A1 Fc, 0.1 μg/ml control Fc, or 100 nM rapamycin and stained with crystal violet. The histogram shows average relative cell growth ± SE from 1–2 experiments with quadruplicate wells. Representative scans of the wells are also shown. (C) Growth of spheroids in Matrigel. Cells plated at low density in Matrigel were grown in a medium containing the indicated concentrations of ephrin-A1 Fc, 0.1 μg/ml control Fc, or 100 nM rapamycin. The resulting spheroids were photographed at the indicated days. The histogram shows average spheroid sizes ± SE from 3 experiments with duplicate wells. Representative spheroids images are also shown. Scale bar = 50 μM. (D) 3D growth in soft agar. Cells plated at low density in soft agar were grown for 3 weeks in a medium containing the indicated concentrations of ephrin-A1 Fc (in μg/ml), 0.1 μg/ml control Fc, or 100 nM rapamycin. The histogram shows average numbers of colonies/photograph ± SEM. Representative photographs are also shown. Scale bar = 100 μM (E) Ephrin-A1 stimulation inhibits S6 kinase and Erk phosphorylation. Cells grown in 10% serum were stimulated for 20 min with the indicated concentrations of ephrin-A1 Fc or Fc as a control. Lysates were probed for phosphorylated S6 kinase and Erk, and reprobed for the corresponding total protein.

conjugated phalloidin. The percentage of retracted cells was quantified from 30 20× microscope fields in 3 independent experiments.

3. Results

3.1. Ephrin-A1 stimulation inhibits PC3 cell growth

It has been recently reported that in neurons ephrin-A-induced activation of EphA receptors inhibits mTORC1 activity [30]. Since mTORC1 is a critical regulator of cancer cell proliferation, particularly in cells in which it is hyperactivated by oncogenic mutations [4,7], we examined whether ephrin-A ligand stimulation inhibits the growth of PC3 prostate cancer cells. The Akt-mTORC1 pathway is hyperactivated in these cells due to a frameshift mutation that abrogates PTEN lipid phosphatase expression [29]. PC3 cells have been widely used to investigate EphA2 signaling pathways because they express high levels of this receptor [18,21,24,31,32].

To activate EphA2, we used a soluble activating form of ephrin-A1 (ephrin-A1 Fc). Ephrin-A1, which is a major ligand for EphA2, is anchored to the plasma membrane by a GPI linkage but soluble forms of this ligand released from the cell surface can also activate EphA2 [33,34]. We found that treatment with ephrin-A1 Fc inhibits PC3 cells 2-dimensional growth on tissue culture plates and 3-dimensional growth in focus formation assays, spheroid formation assays in Matrigel, and colony formation in soft agar (Fig. 1A–D). Ephrin-A1 stimulation also inhibited phosphorylation of S6 kinase at T389 (Fig. 1E), which is a sensitive readout for mTORC1 activity. Thus, ephrin-A1 stimulation can overcome the constitutive activation of mTORC1 caused by loss of PTEN.

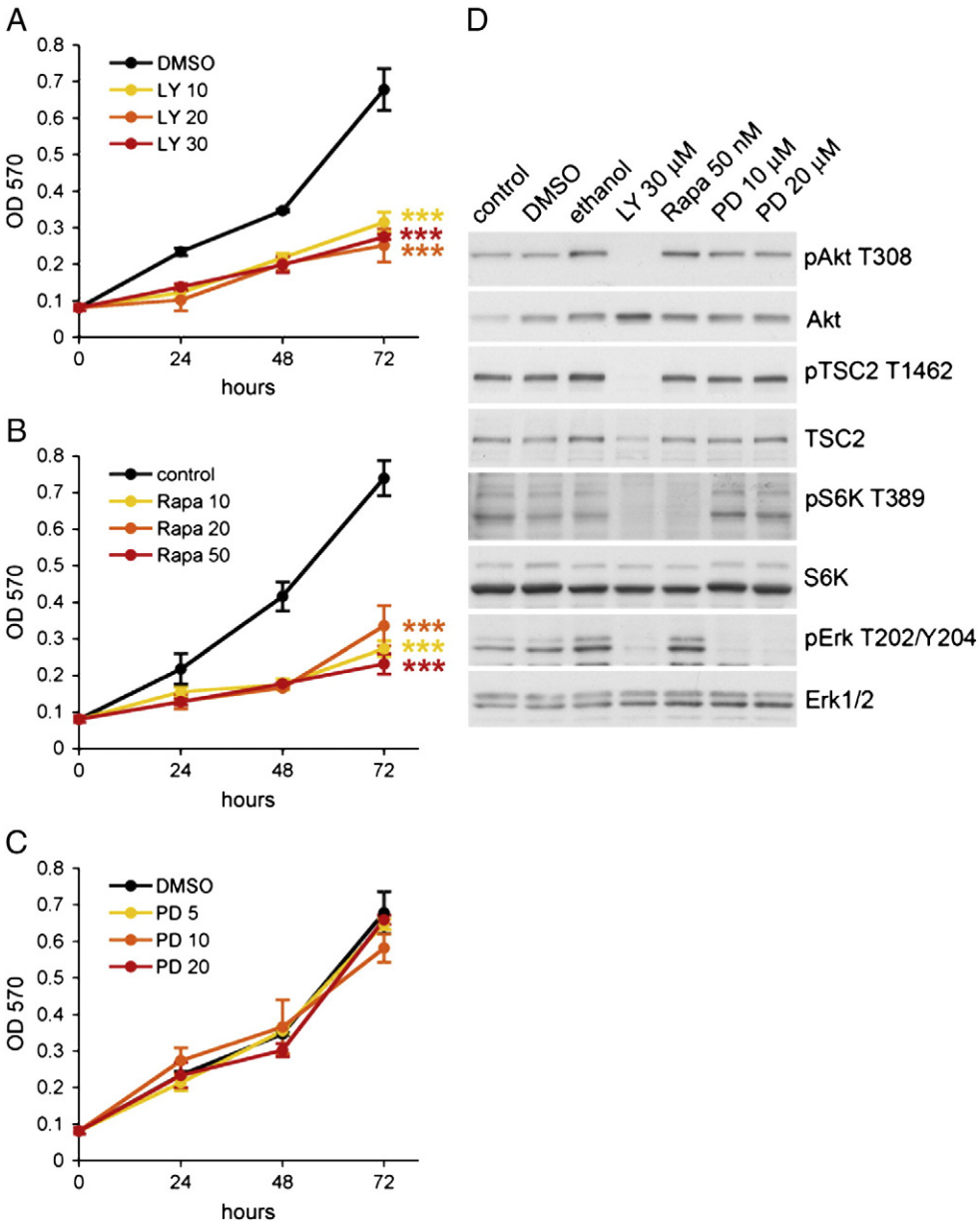

Fig. 2. The Akt-mTORC1 pathway is critical for PC3 cell growth. (A, B, C) Cells were grown in a medium containing 10% FBS and the indicated concentrations of (A) the PI3 kinase inhibitor LY294002 (μM), (B) the mTORC1 inhibitor rapamycin (nM), or (C) the Ras-MAP kinase pathway inhibitor PD98059 (μM). Cell growth was measured using the MTT assay. The graphs show the average absorbance ± SD from duplicate measurements. All the curves were obtained in the same experiment, and the DMSO control curve is the same in (A) and (C). ***P<0.001 compared to DMSO by one-way ANOVA analysis of the 72 hour time points followed by Dunnett's posthoc test. Control indicates vehicle control. (D) Immunoblots verify that the PI3 kinase inhibitor LY294002 inhibits Akt, TSC2 and S6 kinase, the mTORC1 inhibitor rapamycin inhibits S6 kinase, and the Mek inhibitor PD98059 inhibits Erk1/2. In addition, LY294002 also inhibited Erk1/2.

Consistent with previous findings [18], ephrin-A1 also decreased phosphorylation of Erk1/2 at T202 and Y204, indicating inhibition of the Ras-Erk MAP kinase pathway.

Interestingly, ephrin-A1 Fc concentrations that caused high acute EphA2 tyrosine phosphorylation (indicative of activation) also induced marked receptor degradation and, therefore, short-lived EphA2 signaling (Suppl. Fig. S1). In contrast, lower ephrin concentrations caused more prolonged persistence of phosphorylated EphA2 due to lower receptor degradation. This may explain why higher ephrin concentrations resulted in less pronounced growth inhibition (Fig. 1A–D).

To examine the involvement of the Akt-mTORC1 and Ras-MAP kinase pathways in PC3 cell growth, we used chemical inhibitors of these pathways. The PI3 kinase inhibitor LY294002 and the mTORC1 inhibitor rapamycin dramatically reduced PC3 cell growth (Fig. 2A, B, and D). In contrast, the PD98059 Mek inhibitor only slightly decreased growth (Fig. 2C and D), consistent with the fact that the Ras-Erk pathway is not highly activated by oncogenic mutations in PC3 cells. Thus, these cells appear to be critically dependent on the Akt-mTORC1 pathway for their growth, which is consistent with previous findings and the notion that cancer cells become dependent on hyperactivated oncogenic pathways [16,35,36].

3.2. Activation of EphA2 inhibits the Akt-mTOR pathway in PC3 cells

In neurons, ephrin-A stimulation inhibits mTORC1 by decreasing Erk1/2-dependent phosphorylation of TSC2 at S664, which results in increased TSC2 activity [30]. Interestingly, this occurs without inhibition of Akt, a kinase that can also inactivate TSC2 by phosphorylating

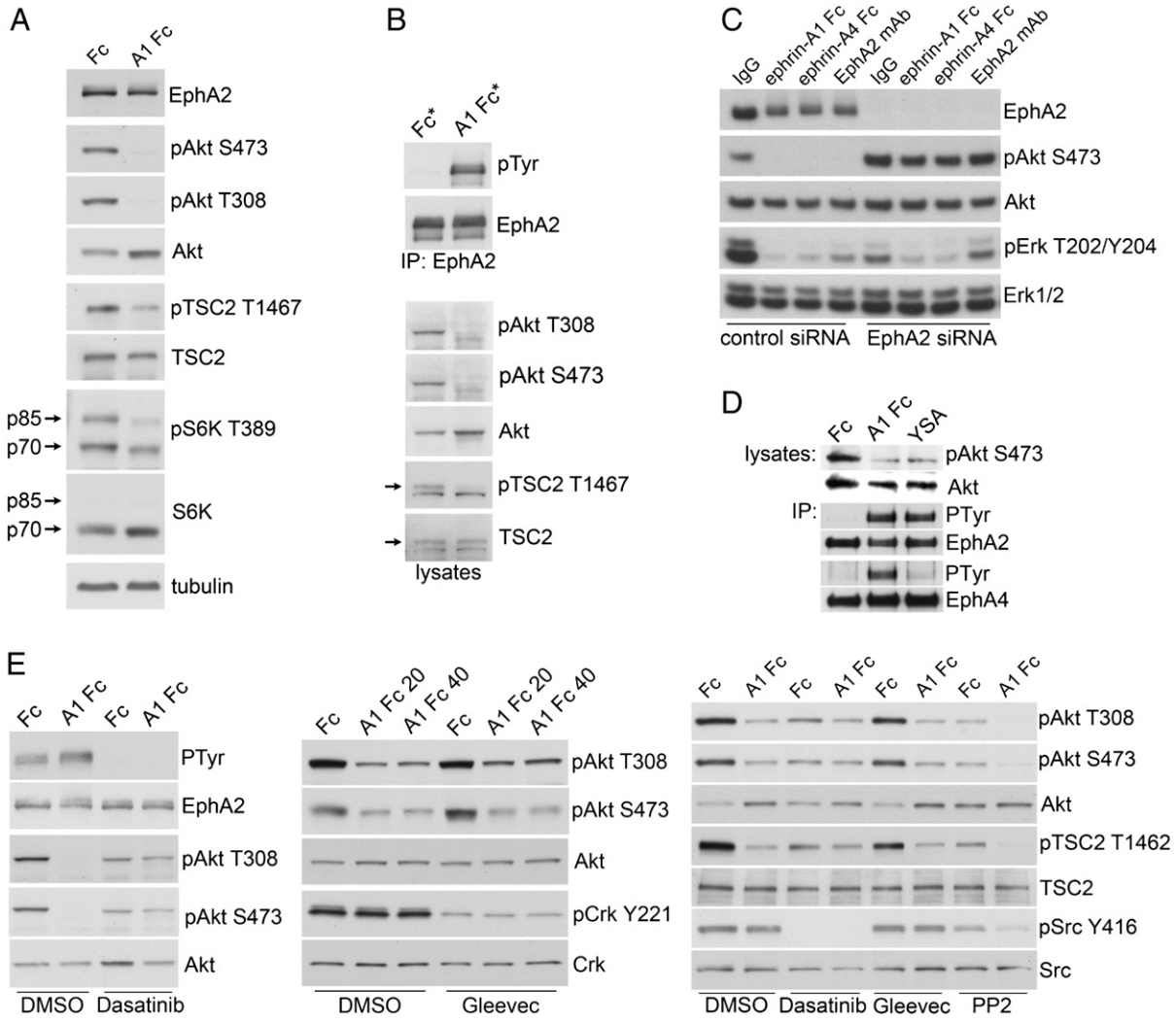

Fig. 3. The EphA2 receptor suppresses the Akt-mTORC1 pathway. (A) Immunoblotting of PC3 cell lysates shows that stimulation with 1 μg/ml ephrin-A1 Fc for 20 min dramatically reduces phosphorylation of Akt at threonine 308 and serine 473, indicating Akt inhibition. Phosphorylation of TSC2 at the Akt target site (threonine 1462) and phosphorylation of S6 kinase at the mTOR target site (threonine 389) were also reduced. Both p70 and p85 S6 kinase isoforms were affected. Cell lysates were probed with the phosphospecific antibodies and reprobed with antibodies to Akt, TSC2, and S6 kinase to verify total protein levels. EphA2 was slightly downregulated by the ephrin-A1 stimulation and probing with anti-tubulin antibodies demonstrates equal loading of the lanes. (B) Stimulation with immobilized ephrin-A1 Fc also inhibits Akt and TSC2 phosphorylation in PC3 cells. Cells were allowed to attach for 15 min on dishes coated with 3 μg/ml ephrin-A1 Fc or Fc as a control (* indicates that the proteins were immobilized on the plate). EphA2 immunoprecipitates were probed with anti-phosphotyrosine antibodies (PTyr) and reprobed for EphA2. Cell lysates were probed as indicated. (C) The 1C1 monoclonal antibody and ephrin-A ligands inhibit Akt phosphorylation only in PC3 cells that express substantial EphA2 levels. Cells transfected with EphA2 siRNA or control siRNA were stimulated for 15 min with 10 μg/ml 1C1 monoclonal antibody, which is a selective EphA2 agonist, 1 μg/ml ephrin-A1 Fc, 1 μg/ml ephrin-A4 Fc, or 10 μg/ml human IgG1 isotype control. Cell lysates were probed with the indicated antibodies. (D) The EphA2-selective agonistic peptide, YSA, decreases Akt phosphorylation. WM793 melanoma cells were stimulated with 1.5 μg/ml clustered ephrin-A1 Fc or control Fc, or 10 μg/ml YSA peptide for 10 min. Cell lysates were probed with the indicated antibodies. EphA2 and EphA4 immunoprecipitates were probed for phosphotyrosine (PTyr) and then reprobed for the respective receptor. (E) Dasatinib (a kinase inhibitor that targets Eph receptors, Abl and Src) prevents loss of Akt phosphorylation induced by ephrin-A1 treatment. In contrast, Gleevec (which targets Abl) and PP2 (which targets Src) are ineffective. Cells were treated with 300 nM dasatinib for 30 min (left panel) or 1 h (right panel); with 10 μM Gleevec for 1 h (middle panel) or 2 h (right panel); or with 10 μM PP2 for 2 h. The cells were then stimulated with 1 μg/ml ephrin-A1 Fc for 15 min in the continued presence of the inhibitors, except for the middle panel where they were stimulated 20 and 40 min in the continued presence of Gleevec.

different sites [37]. In contrast, ephrin-A1 treatment has recently been shown to inhibit Akt phosphorylation in several cancer cell types [28], although increased Akt phosphorylation downstream of EphA2 has also been reported [38]. In PC3 cells treated with ephrin-A1 Fc, we detected a dramatic loss of Akt phosphorylation at both T308 and S473, suggesting decreased Akt activity (Fig. 3A). Indeed, phosphorylation of TSC2 at T1467 and GSK3β at S9 – both of which are Akt target sites – was also reduced (Fig. 3A and data not shown). Furthermore, we observed decreased Akt phosphorylation when PC3 cells were stimulated by contact with immobilized ephrin-A1 Fc to mimic the characteristic mode of Eph receptor activation through contact with ephrins immobilized on adjacent cell surfaces (Fig. 3B). Thus, EphA receptor activation by either soluble or immobilized ephrin-A1 inhibits the Akt-mTORC1 signaling pathway in PC3 cells.

Ephrin-A4 had similar effects as ephrin-A1, consistent with the ability of these ligands to promiscuously activate EphA receptors (Fig. 3C). Activation of EphA2 appears to be sufficient to inhibit Akt because an EphA2-specific activating antibody [39] also caused loss of Akt phosphorylation (Fig. 3C; Suppl. Fig. 2). Furthermore, the YSA peptide – which is also a selective agonist for EphA2 [40] – reduced Akt phosphorylation not only in PC3 cells but also in WM793 melanoma cells (Fig. 3D) [83]. Thus, EphA2 signaling inhibits Akt phosphorylation in different cancer cell types. In addition, siRNA-mediated downregulation of EphA2 abolished the effect of ephrin-A1 on Akt phosphorylation (Fig. 3D), indicating that EphA2 signaling is required for Akt inhibition in PC3 cells. The critical involvement of EphA2 in ephrin-A1-induced Akt inactivation is consistent with a previous report suggesting that EphA2 is the most abundant EphA receptor expressed in PC3 cells [41]. Interestingly, Erk MAP kinases were still inhibited by ephrin-A1 and ephrin-A4 in siRNA-transfected PC3 cells, suggesting the presence of other EphA receptors that can inhibit Erk1/2 but not Akt.

We also found that dasatinib, a potent EphA2 receptor kinase inhibitor originally identified as a Src and Abl kinase inhibitor [38,42], blocked loss of Akt phosphorylation in PC3 cells stimulated with ephrin-A1 Fc, whereas the Abl inhibitor Gleevec and the Src inhibitor PP2 were ineffective (Fig. 3E). This suggests that EphA2 kinase activity is required for Akt inhibition.

3.3. EphA2 signaling can inhibit Akt independently of Ras GTPases

EphA2 signaling inhibits H-Ras, a GTPase that can bind to the p110 catalytic subunit of PI3 kinase and enhance its activity [18,43]. EphA2 may therefore cause Akt inactivation through inhibition of H-Ras and PI3 kinase. However, we found that ephrin-A1 stimulation of the MDA-MB-231 breast cancer cell line, which expresses the constitutively

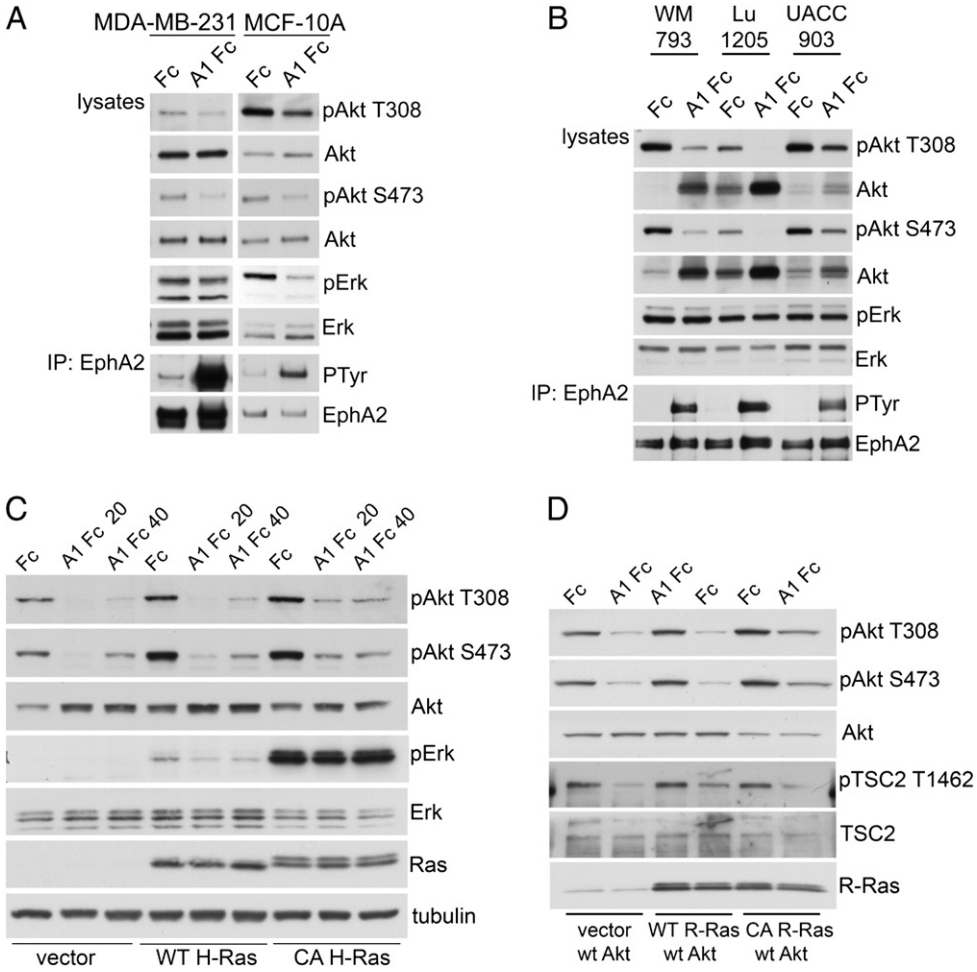

Fig. 4. EphA2 decreases Akt phosphorylation independently of Ras GTPases. EphA2 activation by ephrin-A1 decreases Akt phosphorylation in (A) MDA-MB-231 breast cancer cells and MCF-10A mammary epithelial cells and (B) the indicated melanoma cell lines. Cells were stimulated with 1.5 μg/ml clustered ephrin-A1 Fc or control Fc for 20 min. Lysates were probed as indicated. The bottom two panels show immunoprecipitated EphA2 probed for phosphotyrosine (PTyr) or EphA2. (C) EphA2 decreases Akt phosphorylation independently of H-Ras. PC3 cells transfected with vector control, wild-type H-Ras, or constitutively active H-Ras G12V were stimulated for 20 min or 40 min with 0.1 μg/ml ephrin-A1 Fc or control Fc. Lysates were probed with the indicated antibodies. (D) EphA2 decreases Akt phosphorylation independently of R-Ras. PC3 cells transfected with vector control, wild-type R-Ras, or constitutively active R-Ras G38VY66F together with wild-type Akt1 (at a 9:1 R-Ras to Akt ratio) were stimulated with 0.1 μg/ml ephrin-A1 Fc or control Fc. Lysates were probed with the indicated antibodies.

active K-Ras G13D mutant [44], still inhibits Akt phosphorylation (Fig. 4A). In contrast, Erk1/2 phosphorylation was not affected, as expected because K-Ras G13D activates Erk1/2 and cannot be inhibited. In comparison, ephrin-A1 inhibited both Akt and Erk in the non-transformed MCF-10A mammary epithelial cells, which do not harbor mutated Ras GTPases. Ephrin-A1 stimulation also caused Akt but not Erk1/2 inactivation in several melanoma cell lines expressing the B-Raf V600E mutant, which constitutively activates Erk1/2 (Fig. 4B). Whether Akt inactivation in the melanoma cells treated with ephrin-A1 depends only on EphA2 or also other EphA receptors remains to be determined.

Transfection of wild-type H-Ras or the constitutively active H-Ras G12V mutant increased basal Akt phosphorylation, indicating that activated H-Ras can indeed promote Akt activation in PC3 cells (Fig. 4C). However, neither wild-type nor constitutively active H-Ras blocked Akt inactivation by ephrin-A1. In comparison, the constitutively active H-Ras G12V enhanced basal Erk1/2 phosphorylation in PC3 cells much more than wild-type H-Ras and abolished ephrin-A1-dependent Erk1/2 inactivation. This indicates that inactivation of Ras GTPases by EphA2 can explain Erk1/2 but not Akt inactivation.

EphA2 signaling also inhibits R-Ras, a more distant Ras family member known to activate PI3 kinase but not the Erk MAP kinase pathway [45]. We therefore also expressed constitutively active R-Ras G38VY66F together with low levels of HA-tagged Akt in PC3 cells to preferentially monitor Akt phosphorylation in the transfected cells (representing ~40% of the cells). Expression of constitutively active R-Ras somewhat increased basal Akt phosphorylation but only slightly reduced the ephrin-dependent decrease in Akt phosphorylation detected in cell lysates (Fig. 4D) and in anti-HA antibody immunoprecipitates from cells transfected with HA-tagged Akt (data not shown). These results suggest that inactivation of Ras family GTPases does not play a major role in the loss of Akt phosphorylation downstream of EphA2. Therefore, other pathways must be involved.

3.4. EphA2 signaling can inhibit Akt independently of PI(3,4,5)P3 levels

Integrin-mediated adhesion can increase Akt phosphorylation through PI3 kinase activation [46], and ephrin-A1 Fc stimulation of PC3 cells has been shown to inhibit β1 integrins [21,24]. Thus, EphA2 signaling might decrease Akt phosphorylation indirectly, through inhibition of integrin activity. Consistent with this, we found that Akt phosphorylation dramatically increases in PC3 cells upon attachment to the β1 integrin ligand fibronectin and in WM793 melanoma cells upon attachment to the β3 integrin ligand vitronectin (Fig. 5A). Furthermore, Akt phosphorylation was reduced in these cells by ephrin-A1 stimulation. However, manganese treatment to prevent integrin inactivation only slightly reduced the ephrin-A1-dependent loss of Akt phosphorylation (Fig. 5B). The efficacy of the manganese treatment was confirmed by the observed inhibition of retraction of the cell periphery (Fig. 5B) [21,45]. Treatment with the 9EG7 β1 integrin-activating antibody to maintain β1 integrin activity in PC3 and WM793 cells plated on fibronectin also partially inhibited cell retraction but not ephrin-A1-dependent loss of Akt phosphorylation (Fig. 5C). Hence, loss of integrin-mediated cell substrate adhesion does not play a critical role in Akt inactivation downstream of EphA2.

To determine whether EphA2 may regulate Akt by inhibiting PI3 kinase through other pathways, we examined the HT-29 colorectal cancer and SKOV-3 ovarian cancer cell lines, which respectively express the constitutively active P449T and H1047R PI3 kinase mutants [44]. Ephrin-A1 stimulation decreased Akt and Erk1/2 kinase phosphorylation in these cells (Fig. 6A), suggesting that inhibition of PI3 kinase activity is not essential for loss of Akt phosphorylation downstream of EphA2. We also expressed in PC3 cells a prenylated form of the p110α catalytic subunit of PI3 kinase, which is constitutively active because its farnesylation mediates permanent membrane association [47]. To preferentially monitor Akt phosphorylation in the transfected cells, we also co-expressed low levels of wild-type Akt (Fig. 6B). As expected, we observed enhanced Akt phosphorylation in cells transfected with both Akt and constitutively active PI3 kinase, compared to cells transfected only with Akt. Akt phosphorylation in cells co-expressing constitutively active PI3 kinase was only slightly decreased by treatment with 0.1 μg/ml ephrin-A1 Fc (not shown). However, treatment with 1 μg/ml ephrin-A1 Fc substantially reduced Akt phosphorylation, albeit less than in cells transfected only with Akt (Fig. 6B). This suggests that even the high levels of Akt phosphorylation resulting from concomitant transfection of constitutively active PI3 kinase and Akt in cells lacking PTEN can be overcome by high levels of stimulation of EphA2-dependent pathways. Hence, even if inactivation of PI3 kinase contributed to Akt inactivation downstream of EphA2, other pathways must also be involved. It should also be noted that EphA2 has been reported to activate – rather than inhibit – PI3 kinase [48–50].

Since ephrin-A1 decreases Akt phosphorylation in PC3 prostate cancer cells as well as WM793, LU1205 and UACC903 melanoma cells,

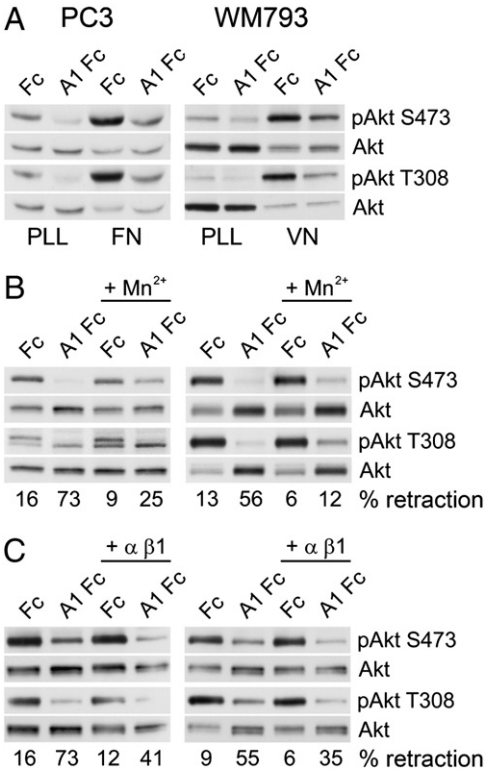

Fig. 5. EphA2 decreases Akt phosphorylation independent of integrin activity. (A) Integrin-mediated adhesion promotes Akt phosphorylation. PC3 cells were plated for 15 min on the β1-integrin ligand fibronectin (FN), or poly-L-lysine (PLL) as a control, and WM793 cells were plated on the β3-integrin ligand vitronectin (VN) or poly-L-lysine in the presence of clustered 7.5 μg/ml ephrin-A1 Fc or control Fc. Lysates were probed with the indicated antibodies. (B) Mn^{2+} treatment does not prevent the loss of Akt phosphorylation induced by ephrin-A1. Cells plated on tissue culture plates were treated for 30 min with 1 mM Mn^{2+} to maintain integrins in an activated state, or left untreated, before stimulation for 30 min with 4 μg/ml ephrin-A1 Fc or control Fc. Lysates were probed with the indicated antibodies. The percentage of retracting cells measured in a parallel experiment in which the cells were plated on poly-L-lysine is indicated below the blots and demonstrates that the Mn^{2+} treatment prevented cell retraction in ephrin-A1 Fc-treated cells, thus verifying maintenance of integrin-mediated adhesion. (C) An integrin-activating antibody does not prevent the loss of Akt phosphorylation induced by ephrin-A1. Cells plated on a fibronectin substrate were treated for 30 min with 20 μg/ml integrin-activating antibody (αβ1) to maintain β1 integrin activation, or left untreated, before stimulation for 30 min with 4 μg/ml ephrin-A1 Fc or control Fc. Lysates were probed with the indicated antibodies. Treatment with the integrin-activating antibody partially prevented retraction of the cell periphery, as shown by the percentage of retracting cells reported below the blot, demonstrating that it prevented some of the loss of integrin-mediated adhesion due to ephrin-A1 Fc stimulation.

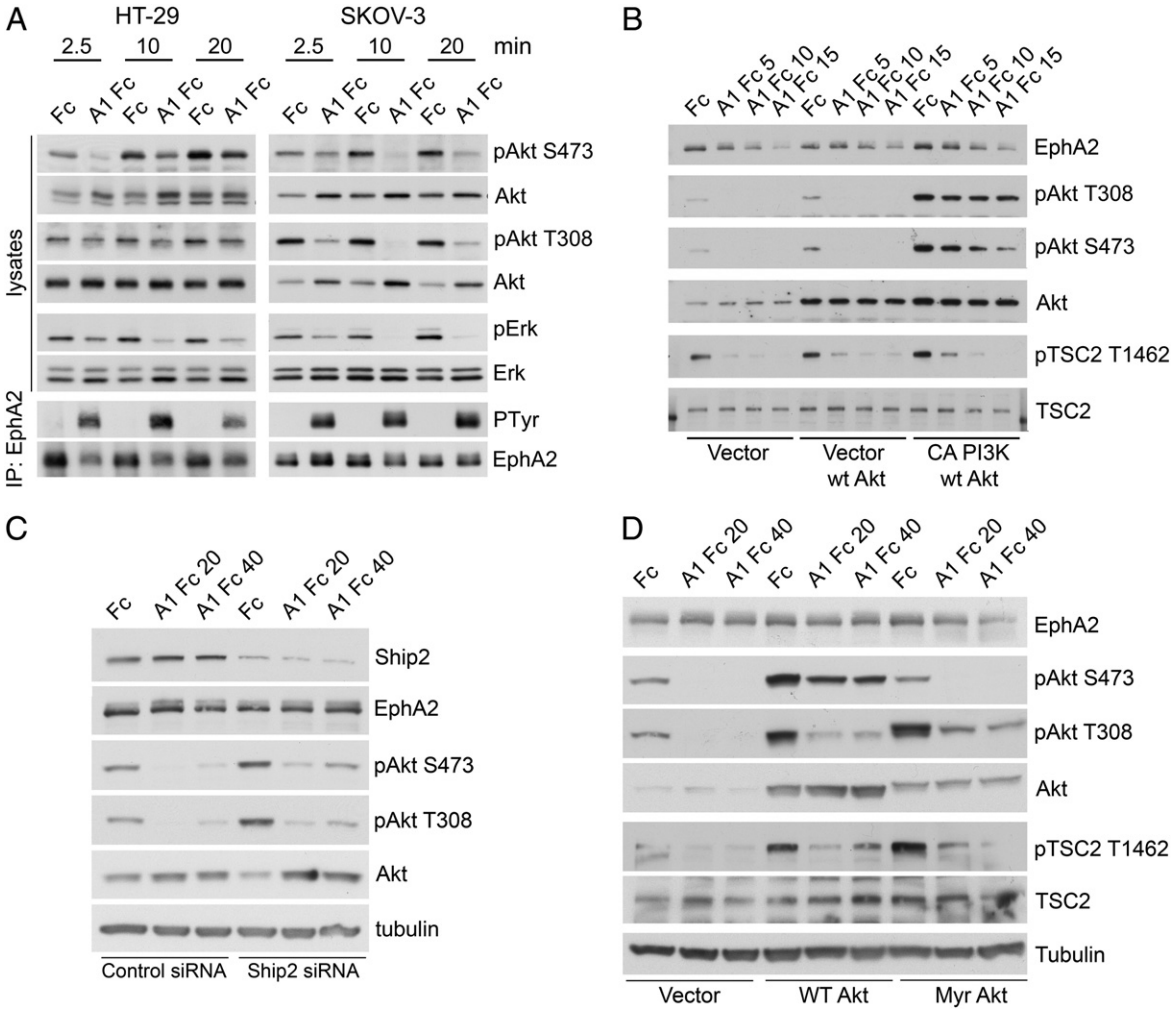

Fig. 6. EphA2 decreases Akt phosphorylation independently of PI3 kinase activity. (A) EphA2 causes Akt dephosphorylation in cells expressing constitutively active PI3 kinase. HT-29 colorectal cancer cells and SKOV-3 ovarian cancer cells were stimulated with 2 μg/ml clustered ephrin-A1 Fc for the indicated times. Lysates were probed as indicated and EphA2 immunoprecipitates were probed with anti-phosphotyrosine antibodies (PTyr) and reprobed for EphA2. (B) EphA2 decreases Akt phosphorylation in cells transfected with constitutively active PI3 kinase. Cells were transfected with vector control, wild-type Akt1, and wild-type Akt1 together with the constitutively active prenylated p110α subunit of PI3 kinase at a 1:9 Akt to PI3 kinase ratio. Cells were then stimulated with 1 μg/ml ephrin-A1 Fc for 5, 10, or 15 min. Lysates were probed with the indicated antibodies. (C) EphA2 decreases Akt phosphorylation independently of the Ship2 lipid phosphatase. Cells were transfected with control siRNA or Ship2 siRNA and stimulated with 0.1 μg/ml ephrin-A1 Fc or Fc as a control for 20 or 40 min and lysates were probed as indicated. (D) EphA2 decreases the phosphorylation of constitutively active, membrane-targeted Akt. Cells were transfected with vector control, wild-type Akt1, and a myristoylated form of Akt1. Cells were then stimulated with 0.1 μg/ml ephrin-A1 Fc or Fc as a control for 20 or 40 min and lysates were probed as indicated.

all of which lack PTEN [29,51], EphA2 does not function by activating PTEN. On the contrary, a recent study in *C. elegans* suggests that Eph receptors may negatively regulate PTEN [52]. However, EphA2 has been shown to associate with Ship2, another lipid phosphatase that can functionally compensate for the loss of PTEN by dephosphorylating PI(3,4,5)P3 [29,50]. We therefore investigated whether enhanced Ship2 activity downstream of EphA2 may be responsible for Akt inhibition in cells treated with ephrin-A1 through a reduction of PI(3,4,5)P3 levels. We found that Ship2 downregulation by siRNA interference increases basal Akt phosphorylation, indicating that Ship2 can indeed regulate Akt activity in PC3 cells (Fig. 6C). However, Ship2 knock down did not prevent ephrin-A1-dependent Akt inactivation (Fig. 6C), suggesting that regulation of Ship2 activity by EphA2 is not critical for Akt inhibition.

Ephrin-A1 treatment also decreased phosphorylation of myristoylated Akt, which is constitutively active due to its permanent membrane localization [53,54] (Fig. 6D). Thus, signaling events occurring downstream of PI3 kinase and independent of PI(3,4,5)P3 levels can lead to Akt dephosphorylation downstream of EphA2.

3.5. Serine/threonine phosphatase activity is required for inhibition of Akt phosphorylation downstream of EphA2

Treatment of PC3 cells with ephrin-A1 can cause an almost complete loss of Akt phosphorylation, similar to that induced by the potent PI3 kinase inhibitor Wortmannin (Fig. 7A). Furthermore, the loss occurs rapidly because Akt phosphorylation is already drastically reduced within 5 min of stimulation with 1 μg/ml ephrin-A1 Fc (Fig. 6B and Suppl. Fig. S3). Interestingly, EphA2 also inhibits Akt phosphorylation when the cells are cultured in a medium without serum (Fig. 7B). The high Akt phosphorylation that is still observed even under serum-free conditions is likely explained by the high PI(3,4,5)P3 levels due to lack of PTEN expression. In contrast, activation of various growth factor receptors is presumably very low in the absence of serum, resulting in very low activity of PI3 kinase upstream regulatory pathways. Thus, it seems unlikely that EphA2 might decrease Akt phosphorylation by inhibiting a pathway upstream of Akt. Rather, a plausible explanation of our findings is that EphA2 regulates a serine/threonine phosphatase that can dephosphorylate Akt.

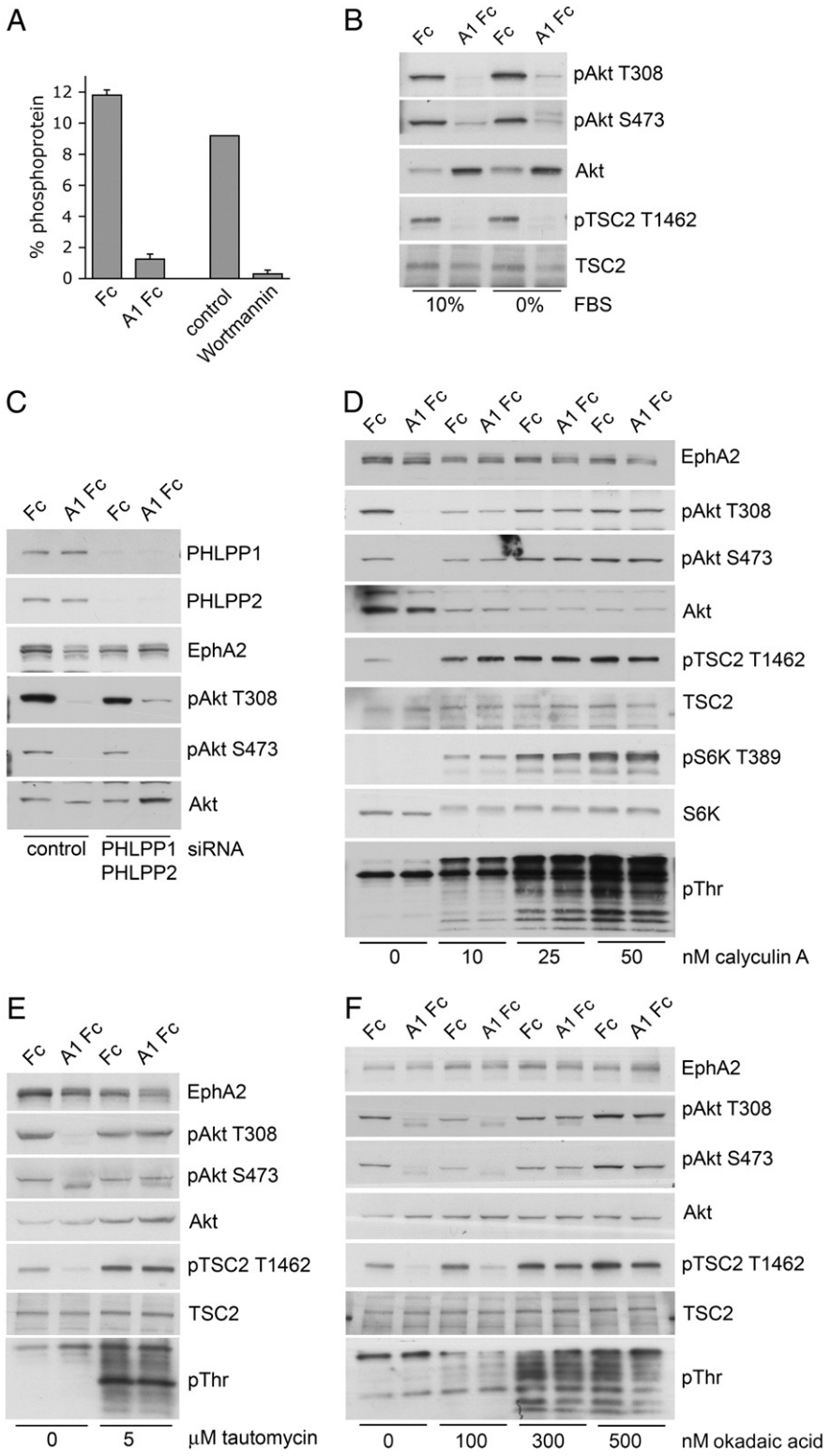

Fig. 7. Serine/threonine phosphatase activity is required for EphA2-dependent Akt dephosphorylation. (A) Ephrin-A1 decreases Akt phosphorylation almost as much as the potent PI3 kinase inhibitor Wortmannin. The fraction of Akt phosphorylated at S473 was measured in cells stimulated for 30 min with 1 μg/ml ephrin-A1 Fc or Fc as a control and in cells treated with 20 nM Wortmannin for 30 min or left untreated (–) using the MesoScale technology. The histogram shows averages ± SD from triplicate measurements for ephrin-A1 and duplicate measurements for Wortmannin. (B) Ephrin-A1 Fc stimulation similarly inhibits Akt phosphorylation in the presence and in the absence of FBS. Cells grown in 10% FBS or starved overnight without serum were stimulated for 15 min with 1 μg/ml ephrin-A1 Fc or Fc as a control. Lysates were probed with the indicated antibodies. (C) EphA2 causes Akt dephosphorylation independently of PHLPP phosphatases. Cells were transfected with control siRNA or siRNAs targeting both PHLPP1 and PHLPP2 phosphatases and stimulated for 15 min with 0.1 μg/ml ephrin-A1 Fc or Fc as a control. Lysates were probed with the indicated antibodies. (D) Calyculin prevents ephrin-A1-dependent Akt dephosphorylation. PC3 cells were incubated for 30 min with the indicated concentrations of the phosphatase inhibitor calyculin, which similarly inhibits both PP1 and PP2A phosphatases. The cells were then stimulated for 15 min with 1 μg/ml ephrin-A1 Fc or Fc as a control. Lysates were probed with the indicated antibodies. Blotting with anti-phospho-threonine antibodies (PThr) demonstrates overall serine/threonine phosphatase inactivation. (E) Tautomycin prevents ephrin-A1-dependent Akt dephosphorylation. PC3 cells were incubated for 4 h with the phosphatase inhibitor tautomycin, which preferentially inhibits PP1 over PP2A. The cells were stimulated and analyzed as in (D). (F) Okadaic acid prevents ephrin-A1-dependent Akt dephosphorylation only when used at high concentrations. PC3 cells were incubated for 4 h with the phosphatase inhibitor okadaic acid, which preferentially inhibits PP2A over PP1. The cells were stimulated and analyzed as in (D).

Several serine/threonine phosphatases could function with EphA2 to inactivate Akt. For example, PHLPP1 and PHLPP2 are two widely expressed phosphatases known to dephosphorylate S473 of Akt [55,56]. However, siRNA-mediated knockdown of these phosphatases did not prevent EphA2-dependent Akt dephosphorylation in PC3 cells (Fig. 7C). Thus, PHLPP phosphatases do not play a critical role in Akt inactivation by EphA2.

To examine the involvement of PP1 and PP2A, two very abundant phosphatases responsible for the dephosphorylation of many cellular proteins [57–60], we examined the effects of calyculin. This inhibitor, which targets both PP1 and PP2A [61–63], completely blocked Akt, TSC2, and S6 kinase dephosphorylation in PC3 cells treated with ephrin-A1 (Fig. 7D). Calyculin also inhibited ephrin-A1-induced Akt dephosphorylation in WM793 and Lu1205 melanoma cells (Suppl. Fig. 4), indicating that this effect is not limited to PC3 cells. Inhibition of Akt dephosphorylation was observed even at the low calyculin concentration of 10 nM, which only slightly affected detectable overall protein threonine phosphorylation. Interestingly, calyculin did not detectably increase the basal level of Akt phosphorylation in control cells not stimulated with ephrin-A1. This suggests that Akt is not constitutively dephosphorylated by a calyculin-sensitive phosphatase in PC3 cells, but becomes a target of the phosphatase when EphA2 is activated by ephrin-A1. Therefore, EphA2 activation by ephrin-A1 triggers Akt dephosphorylation by a calyculin-sensitive serine/threonine phosphatase.

To discriminate between PP1 and PP2A, we used the more selective inhibitors tautomycin, and okadaic acid [62,64–66]. Tautomycin blocked Akt dephosphorylation induced by ephrin-A1 at the concentration of 5 μM (Fig. 7E), which preferentially inhibits PP1 over PP2A [62,64]. Okadaic acid also blocked Akt dephosphorylation induced by ephrin-A1, but only at the high concentrations (300 nM) that have been reported to inhibit not only PP2A but also PP1 [64,65,67] (Fig. 7F). In contrast, lower concentrations that should preferentially inhibit PP2A (100 nM) did not block Akt dephosphorylation. These results implicate a PP1-like phosphatase, or another phosphatase that is sensitive to calyculin and tautomycin but not to low concentrations of okadaic acid, in Akt dephosphorylation downstream of EphA2.

3.6. EphA2 phosphorylation is constitutively regulated by a serine/threonine phosphatase

It has been recently reported that Akt phosphorylates EphA2 at S879 and that this phosphorylation can be detected with an antibody recognizing Akt substrate motifs [28]. We found that treatment with calyculin, tautomycin or high concentrations of okadaic acid substantially increases EphA2 recognition by the anti-Akt substrate antibody in ephrin-A1-stimulated as well as control cells (Fig. 8). The increased EphA2 phosphorylation in cells treated with the phosphatase inhibitors suggests that EphA2 continuously undergoes dephosphorylation by a phosphatase with the same inhibitor sensitivity profile as the phosphatase that dephosphorylates Akt. This further supports a functional interplay between EphA2 and one or more serine/threonine phosphatases.

4. Discussion

Both tumor promoting and tumor suppressing activities have been reported for Eph receptors, and the molecular mechanisms responsible for one versus the other outcome are under intense investigation. EphA2 is the Eph receptor that has attracted most attention in the cancer field. Its expression is upregulated downstream of the Ras-MAP kinase pathway, which is often hyperactivated by oncogenic mutations [17,68]. This may explain the high EphA2 levels found in many cancer types. Ephrin-dependent activation of EphA2 can in turn suppress the Ras-MAP kinase pathway by activating the Ras GTPase-activating protein, p120RasGAP [17,45,69]. We report here that, remarkably, the EphA2 receptor also suppresses another major oncogenic pathway, the Akt-mTORC1 pathway through a novel signaling mechanism.

While our work was in progress, ephrin-A1-dependent activation of EphA2 has been reported to decrease Akt phosphorylation in PTEN-deficient cancer cells [28]. However, the mechanism involved was not elucidated. It has also been recently shown that ephrin-A-mediated activation of neuronal EphA receptors inhibits mTORC1 by decreasing Erk1/2-dependent phosphorylation of TSC2 without affecting Akt phosphorylation [30]. Taken together, these findings suggest that different EphA receptors can inactivate mTORC1 by using different mechanisms. According to a recent report, EphB receptors can also inhibit Akt [70].

Our evidence suggests that the major mechanism by which EphA2 reduces Akt phosphorylation in PC3 cells does not involve inhibition of upstream regulatory pathways. Neither expression of constitutively active Ras proteins nor blocking integrin inactivation prevented ephrin-A1-induced loss of Akt phosphorylation, suggesting that the main underlying mechanism does not rely on decreased PI3 kinase function due to inhibition of Ras or integrin activity. Furthermore, neither the lack of PTEN in PC3 and melanoma cells nor the PI3 kinase activating mutations in SKOV-3 and HT-29 cells prevented ephrin-A1-induced inactivation of Akt. This is in contrast to the strong effect of K-Ras and B-Raf oncogenic mutations or transfection of constitutively active H-Ras, all of which abolished ephrin-A1-dependent inactivation of Erk1/2. Hence, constitutively activating mutations of upstream regulators can prevent Erk1/2 but not Akt inactivation downstream of EphA2. We also found that although Ship2 knock down in PC3 cells increases basal Akt phosphorylation, it did not prevent Akt inactivation

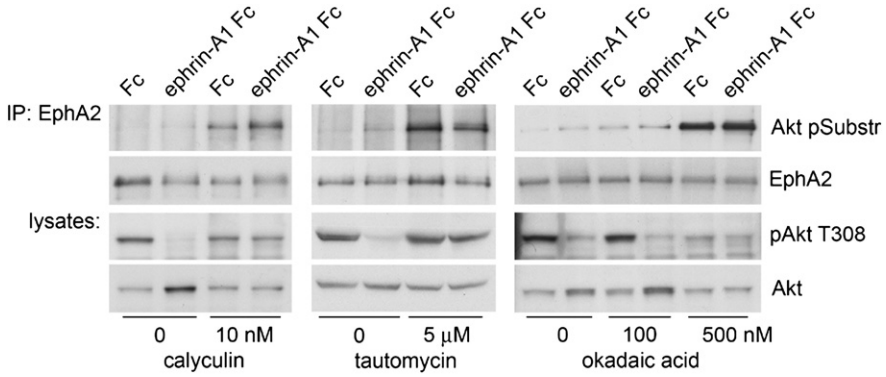

Fig. 8. A PP1-like phosphatase reduces EphA2 serine/threonine phosphorylation. PC3 cells pre-treated with the indicated serine/threonine phosphatase inhibitors were stimulated with 1 μg/ml ephrin-A1 Fc for 15 min. EphA2 immunoprecipitates were probed by immunoblotting with an antibody to the phosphorylated Akt substrate motif and reprobed with EphA2 antibodies. Akt phosphorylation in cell lysates was also monitored to verify inhibition of phosphatase activity. Calyculin, tautomycin and high levels of okadaic acid dramatically increase EphA2 phosphorylation even in Fc-treated control cells.

by EphA2. Our experiments also show that EphA2 can decrease phosphorylation of myristoylated Akt, suggesting that EphA2-dependent inhibition of Akt does not occur through a reduction in its membrane association. In addition, we did not detect EphA2-induced downregulation of PDK1 levels or phosphorylation at S241 (data not shown), suggesting that EphA2 also does not inhibit PDK1 [71].

Interestingly, a recent analysis of the signaling networks activated downstream of another Eph receptor, EphB2, has suggested that protein phosphatases are important effectors of Eph receptors [72]. In agreement with this, the low-molecular-weight protein tyrosine phosphatase functions downstream of EphA2 in cancer cells [73]. We therefore examined whether EphA2 may promote Akt dephosphorylation by a serine/threonine phosphatase. The recently discovered PHLPP1 and PHLPP2 phosphatases are known to dephosphorylate S473 of Akt and their regulation is poorly understood [55,56]. Knock down of both PHLPP1 and PHLPP2, however, did not prevent Akt dephosphorylation by EphA2.

PP1 and PP2A are the major serine/threonine phosphatases found in eukaryotic cells [57–60]. They dephosphorylate a multitude of cellular proteins, and both are capable of dephosphorylating Akt. The substrate selectivity of PP1 and PP2A is mainly controlled through protein–protein interactions. PP1-interacting proteins, some of which are also substrates, contain consensus binding motifs such as RVxF and (S/G)ILK [57,74,75]. Interestingly, the EphA2 kinase domain contains a similar GMLK sequence in a loop within the N-terminal lobe of the kinase domain, although it is not known whether this motif is functional and whether its PP1 binding ability may be affected by receptor activation. On the other hand, a RVDF sequence also found in the N-terminal kinase lobe is unlikely to bind PP1 because it is not in a loop and the aspartic acid at the variable position likely does not support PP1 binding. While both PP1 and PP2A are known to associate with Akt and have been implicated in its dephosphorylation [59,60,62,65,76,77], our inhibitor selectivity profile suggests the involvement of a PP1-like phosphatase in Akt inhibition by EphA2 in PC3 and several other cancer cell lines, consistent with the idea that PP1 may play a preferential role in Akt dephosphorylation in cells of epithelial origin [65].

The phosphatase inhibitors did not affect Akt phosphorylation under basal conditions, suggesting that Akt is dephosphorylated by a PP1-like phosphatase only when the cells are stimulated with ephrin-A1. Alternatively, feedback loops may keep Akt phosphorylation low when the inhibitors are present. In contrast, phosphatase inhibition greatly enhanced EphA2 phosphorylation at the Akt substrate motif, suggesting that a continuous functional interplay between EphA2 and a phosphatase keeps phosphorylation of this site low. Interestingly, a similar scenario has been reported for the Ron receptor tyrosine kinase, which is phosphorylated by Akt on S1394 near the carboxy terminus [78]. This phosphorylation is also increased by phosphatase inhibitors and promotes the binding of PP1, which in turn dephosphorylates the Ron receptor. Therefore, a plausible model is that EphA2 facilitates the functional interaction of a PP1-like phosphatase with activated Akt. Consistent with this model, overexpressed EphA2 has been shown to colocalize with activated Akt at the leading edge of polarized cells [28]. Furthermore, EphA2 activation does not appear to increase overall cellular levels of threonine-phosphorylated proteins detected by immunoblotting and measurements in extracts of PC3 cells stimulated with ephrin-A1 did not reveal overall decreases in phosphatase activity (data not shown). These findings suggest a localized rather than global effect of EphA2. However, we could not conclusively identify the specific phosphatase(s) involved because siRNA-mediated downregulation of PP1 or PP2A catalytic subunits caused extensive PC3 cell death (data not shown). Since both phosphatases can associate with many regulatory subunits that direct them to different substrates [57–60], and since EphA2 itself may fulfill the function of a PP1 regulatory protein, additional work will be required to identify the mechanism of Akt dephosphorylation downstream of EphA2 and the specific phosphatase involved. Other serine/threonine phosphatases, such as PP4 or PP6, cannot be completely discounted because they also have some sensitivity to the inhibitors used [79].

The ability of EphA2 to cause Akt dephosphorylation may be also affected by the cellular context, because we did not detect substantial Akt dephosphorylation in a few of the EphA2-expressing cell lines examined, including U251 glioma cells, MCF7 breast cancer cells, and ES2 and HEYA8 ovarian cancer cells (data not shown). Ephrin-A1-dependent stimulation of EphA2-transfected B16 melanoma, LNCaP prostate cancer, COS and 293 HEK cells, which do not endogenously express the receptor, also did not cause Akt dephosphorylation. Moreover, EphA2 was shown to activate Akt in pancreatic cancer cells [38]. It will be interesting to elucidate the mechanisms underlying this differential responsiveness.

Loss of PTEN is particularly critical for prostate cancer development and malignancy due to hyperactivation of the Akt-mTORC1 pathway [12,13,80,81]. Akt can also promote cell survival, proliferation and invasiveness in many other types of cancer and its oncogenic effects can be enhanced by concomitant hyperactivation of the Ras-MAP kinase pathway [16,82]. Our data suggest that EphA2 activation can overcome the effects of oncogenic mutations in the PI3 kinase-PTEN-Akt pathway. EphA2 acutely inhibits Akt-mTORC1 as effectively as LY294002, Wortmannin or rapamycin, and also inhibits the Ras-MAP kinase pathway. However, EphA2-dependent inhibition of cell growth is lower than that of the chemical inhibitors. This is likely due to the transient nature of the EphA2 signals, because ephrin-A stimulation triggers EphA2 internalization and degradation, which ultimately results in at least partial restoration of Akt and Erk1/2 phosphorylation. Recent data suggest that lack of PTEN may even accelerate EphA2 degradation [52]. Ephrin-induced EphA2 degradation likely explains our observation that ephrin-A1 has a more pronounced effect on PC3 cell growth at concentrations that do not maximally activate EphA2 because, over prolonged periods, lower ephrin concentrations result in higher steady-state levels of activated EphA2 due to decreased receptor degradation. Thus, EphA2-based anti-cancer treatments could be more effective if activation of EphA2 forward signaling can be achieved without inducing drastic receptor downregulation.

5. Conclusions

This work shows that activation of the EphA2 receptor tyrosine kinase by ephrin-A ligands in cancer cells can inhibit a major oncogenic signaling pathway, the Akt-mTORC1 pathway. This tumor suppressor activity of EphA2 was observed even when Akt and mTOR are hyperactivated due to mutations in the PTEN lipid phosphatase and in cells harboring constitutive activating mutations of PI3 kinase, Ras or B-Raf. Our data suggest that crosstalk of EphA2 with a serine/threonine phosphatase plays a critical role in ephrin-A-dependent Akt inactivation.

Supplementary materials related to this article can be found online at doi:10.1016/j.cellsig.2010.09.004.

Conflict of interest

ZX and DAT are employees of MedImmune/AstraZeneca. EBP received a grant from MedImmune/AstraZeneca that partially supported this research. The other authors declare no potential conflict of interest.

Contributors

All authors designed and interpreted some of the experiments. NYY performed most of the experiments involving PC3 cell transfections and phosphatase inhibitor treatments and helped write the

manuscript. CF performed the PC3 cell growth experiments. MR performed all the experiments with cells other than PC3 cells and the experiments to investigate the involvement of integrins. ZX performed initial experiments identifying inhibition of Akt phosphorylation by EphA2, experiments with the 1C1 antibody, and phosphatase inhibitor experiments. FV performed initial experiments identifying inhibition of Akt phosphorylation by EphA2, including the ephrin concentration, time and serum dependence of the effects. DAT and EPB conceived the project and designed and interpreted the experiments. EBP wrote the manuscript with help from NYY and DAT. All authors read and approved the final manuscript.

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